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13. ABSTRACT (Maximum 200 Words)
Prostate cancer (PCa) is the most common invasive malignancy and second leading cause of cancer death in men in the United States. Up till now, hormone ablation therapy is the major way to treat PCa. Such therapy only causes a temporary regression and tumor growth resumes within 6-18 months. Therefore, better androgen blockage is not the answer for treating PCa. Rather, research efforts should focus on the therapeutic agents that will inhibit growth factor signaling pathways thereby inhibit growth. A large number of studies have pointed out that inositol hexaphosphate (IP6) could have beneficial effect on variety of cancers. The specific aims of this proposal are to determine (1) the in vivo effect of IP6 on the growth of PCa (2) the efficacy of IP6 in inhibiting growth factor-induced DNA synthesis of the PCa cells in vitro, and (3) the molecular mechanisms by which IP6 inhibits growth of PCa cells. The information we obtain from these experiments will provide a better understanding of the potential role of IP6 in the prevention of growth of PCa cells. This information will lead to more effective PCa prevention and treatment strategies in human that might prolong the longevity of men with prostate cancer.

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INTRODUCTION:

Prostate cancer is the most common invasive malignancy and second leading cause of cancer death in men in the United States and many other parts of the world. Up till now, hormone ablation therapy is the major way to treat prostate cancer. Such therapy only causes a temporary regression and tumor growth resumes within 6-18 months. It is now well established that aberrant expressions of mitogenic growth factors and their receptors are responsible for unregulated growth of the prostate cancer. Once autocrine growth factor loops are operative, prostate cancer progresses to an androgen-independent state. It is uniformly fatal because no systemic therapy currently exists that inhibit growth of androgen-independent prostate cancer. Therefore better androgen blockade is not the answer for treating prostate cancer. Rather, research efforts should focus on the therapeutic agents that will inhibit growth factor signaling pathways thereby inhibit growth. While many new classes of cancer chemopreventive agents are being evaluated in clinical trials for other malignancies, little success has been achieved in terms of prostate cancer prevention. During the past several years, a large number of studies have pointed out that inositol hexaphosphate (IP6), the most abundant phosphorylated inositol present in beans, cereal grains, lentils and legumes, could have beneficial effect on variety of cancers. The underlying hypothesis driving our work is that unregulated expression of mitogenic growth factors are responsible for carcinogenesis of the prostate gland and IP6 can prevent such development by inhibiting growth factor-induced signal transduction. Therefore, IP6 could be a potential agent for the prevention and treatment of prostate cancer. The specific aims of this project are to examine (1) the in vivo effects of IP6 on the growth of prostate cancer (2) the efficacy of IP6 in inhibiting growth factor-induced DNA synthesis of prostate cancer cells in vitro, and (3) to determine the molecular mechanisms by which IP6 inhibits growth of prostate cancer cells.

BODY:

In my proposal under the "Statement of Work", I proposed that my first task would be to determine the in vivo effects of inositol hexaphosphate (IP6) on the growth and development of prostate cancer in TRAMP mice. To test the efficacy of IP6 in preventing prostate cancer growth, 32 male TRAMP mice of 4 weeks of age were treated with 1, 2 and 4% IP6 or without IP6. As seen in Figure 1A, IP6 dose-dependently decreased prostate tumor growth over 32 weeks of treatment. Although, we observed a dose-dependent decrease in tumor growth, significant inhibition was only observed in 4% IP6-treated groups. In control diet groups, tumor was very large in size and was exclusively in the prostate gland whereas seminal vesicle was normal (Fig. 1B). IP6 (4%) treatment inhibited such tumor growth in the prostate (Fig 1C). At this point, we are evaluating the histopathology of IP6 treated and untreated groups and results will be reported soon.

KEY RESEARCH ACCOMPLISHMENTS:

- In vivo treatment of IP6 to TRAMP mice is complete (Task 1)
- Mechanism of growth inhibition by IP6 has been resolved (Task3)
- Efficacy of IP6 in inhibiting GFs induced DNA synthesis is partly complete (Task 2)

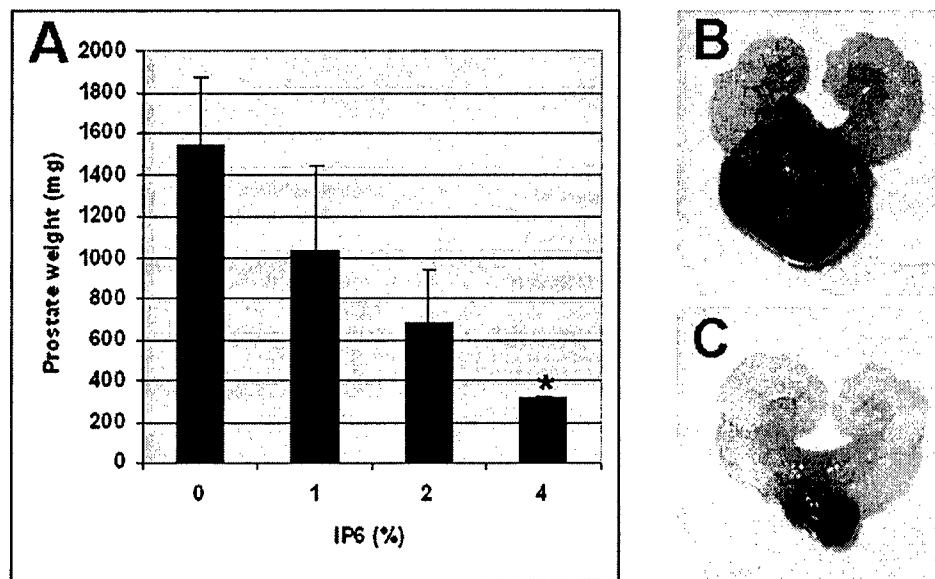(Please see the results in next few pages; Figure 1-8)

Figure 1. Effects of IP6 treatment on TRAMP prostate growth in vivo. A: Weight of prostate tumor after 32 weeks of IP6 treatment in vivo. IP6 induced a dose-dependent decrease in prostate tumor growth. B: a representative photograph from a control TRAMP tumor at 36 weeks of age. C: a representative photograph of a 4% IP6-treated TRAMP prostate. * indicates significant differences from control. Data represents the results of 8 animals per group.

Although, we observed a dose-dependent inhibition of prostate tumor growth, these doses of IP6 did not cause any overt toxicity in these animals. As we observed, there is no significant change in the body weight (Fig 2 A) or in five vital organs, heart, kidney, liver, lung and testis (Fig. 2 B). We are currently looking into the histopathology of these organs to confirm that IP6 did not cause any damage to these vital organs over 8 months of treatment.
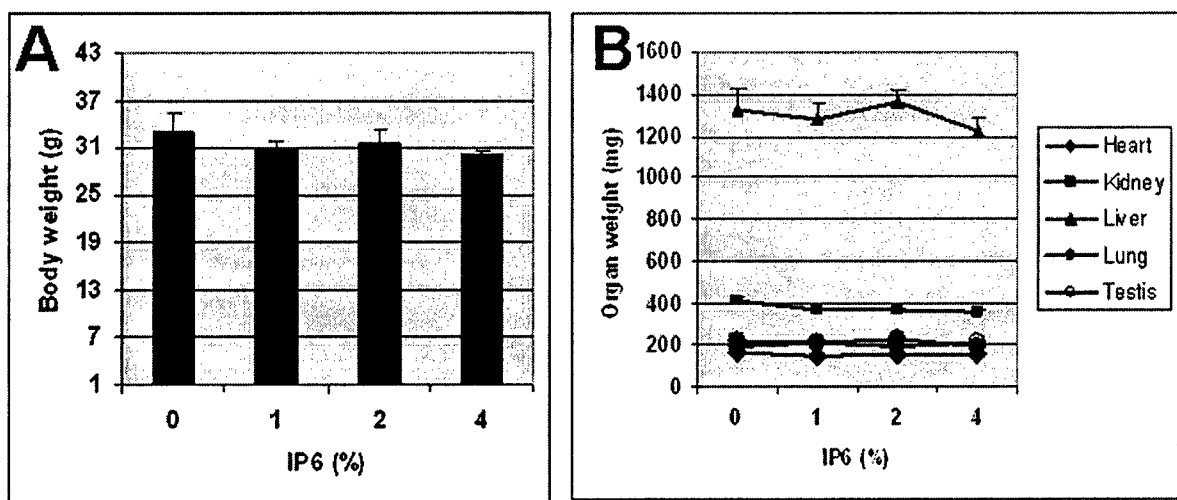
Figure 2. Effect of IP6 on body weight and organ weights in TRAMP mice. A: Body weights at 36 weeks-old TRAMP mice after various doses of IP6 treatment. B: Various organ weights at 36 weeks of age after various doses of IP6 treatment.

To examine the in vivo effect of IP6 at the molecular level, we examined the effect if IP6-induced growth inhibition in TRAMP cells. First, we examined whether IP6 inhibits TRAMP prostate cancer cells growth. As we see in Figure 3A there is a dose-dependent decrease in cell growth in both TRAMP C1 and C2 cells. Significant inhibition occurred by 2 mM of IP6. Similarly, we also observed that IP6 also decreased the DNA synthetic ability of these cells dose-dependently, and by 2 mM concentration BrdU labeling was decreased approximately 50% (Fig 2B), suggesting that similar to in vivo situation, IP6 can inhibit TRAMP prostate cancer cell growth in vitro.
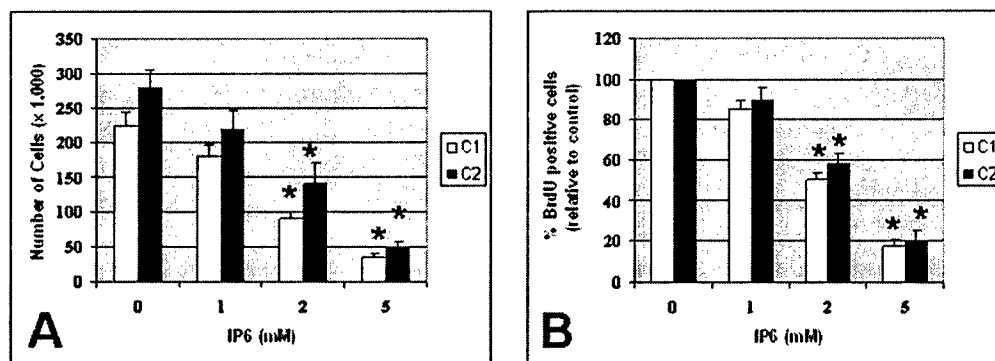

Figure 3. Effects of IP6 on growth and DNA synthesis of TRAMP cells in complete growth media. A: Dose-dependent growth inhibition of TRAMP C1 and C2 cells after 3 days of culture. B: Dose-dependent inhibition of DNA synthesis (BrdU incorporation) in TRAMP C1 and C2 cells after 3 days. * indicates significant differences compared to their respective controls.

We also observed that IP6 induces G0/G1 arrest in TRAMP C2 cells as early as 24h of treatment (Fig 4). As a result the S-phase decreases significantly. It was also evident in earlier experiment in BrdU incorporation study (Fig. 3B).
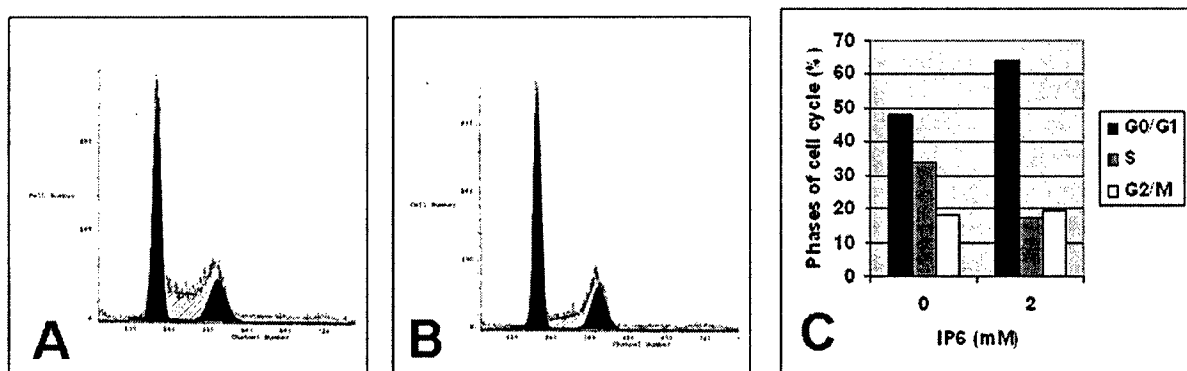
Figure 4. Effects of IP6 on cell cycle progression in TRAMP cells. A: Flow cytometric data showing various phases of cell cycle in TRAMP cells with (B) or without (A) IP6 treatment for 24h. C: quantitative data comparing the cell cycle between IP6 treatment and without treatment.

Since, we observed the cell cycle arrest at G0/G1, we decided to examine some of the dominant players of this phase of cell cycle. As we see in Figure 5A, the level of PCNA, cyclin D1 and E2F1 decreased dose-dependently. The dramatic effect was observed in cyclin D1, where even 1 mM IP6 caused almost complete inhibition of this protein expression. We are currently looking at the promoter of this gene to determine the molecular regulation of cyclin D1 by IP6. Using PCNA promoter-luciferase construct, we observed that IP6 induced a significant decrease in the PCNA promoter activity (Fig. 5B). This result suggests that IP6 can inhibit prostate cancer growth by down regulating the PCNA transcription and by decreasing PCNA protein expression. We do not know whether the translocation of PCNA is also associated with the IP6 treatment.
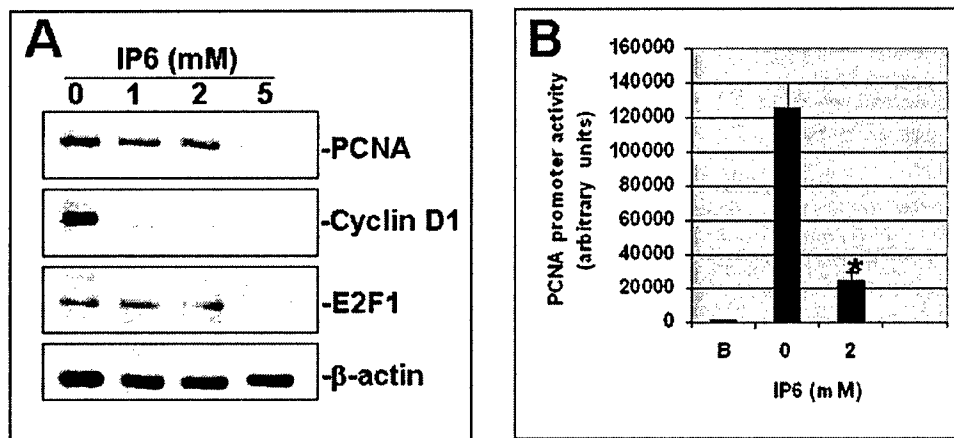

Figure 5. Effects of IP6 on cell cycle regulators of G0/G1 phase in TRAMP cells. A: Western blots showing the levels of PCNA, Cyclin D1 and E2F1 after various doses of IP6 treatment. B: PCNA promoter activity with or without IP6 treatment in TRAMP C2 cells. * indicates significant differences compared to the respective control.

In search of molecular mechanisms of IP6 induced growth inhibition of TRAMP cancer cells, we discovered that IP6 dose-dependently decreased telomerase activity (Fig. 6). We also observed that this decrease in telomerase activity is not TRAMP cell specific, it also occurs in human prostate cancer cells, LNCaP (Fig. 6C). Using quantitative estimation, we observed that with 2 mM IP6 caused 50% inhibition of telomerase activity by 3 days of treatment and with 5 mM concentration it further reduces to approximately 20% of the control levels (Fig. 6D). These results clearly suggest for the first time that IP6 can inhibit telomerase activity in prostate cancer cells and thereby inhibits prostate cancer ells ability to replicate indefinitely.
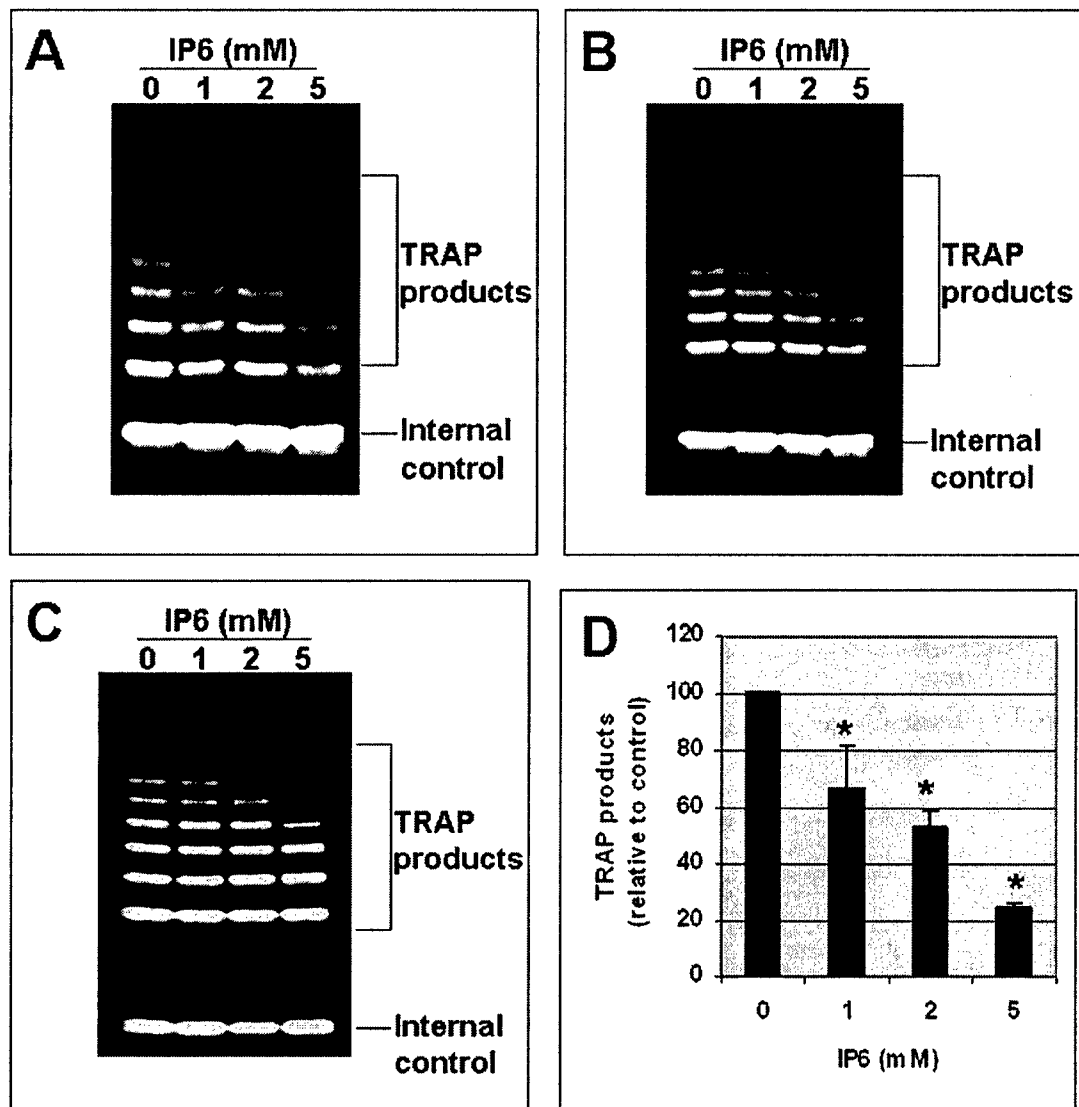
Figure 6. Effects of IP6 on the telomerase activity in TRAMP and human prostate cancer cells. A: TRAP assay showing the levels telomerase activity in IP6 treated and untreated TRAMP Cl cells. B: TRAP assay showing the levels telomerase activity in IP6 treated and untreated TRAMP C2 cells. C: TRAP assay showing the levels telomerase activity in IP6 treated and untreated LNCaP cells. D: Quantitative estimation of telomerase activity with various doses of IP6 in TRAMP C2 cells. * indicates significant differences compared to the respective control.

Since we observed that the telomerase activity decreases in response to IP6 treatment in TRAMP and human prostate cancer cells, we examined the message level of TERT, catalytic subunit of telomerase, expression of TERT is tightly associated with the telomerase activity. Using RT-PCR, we examined the mRNA level of TERT and normalized with GAPDH expression. As seen in Fig. 7A, there is a dose-dependent decrease in the expression of TERT mRNA, suggesting decrease in telomerase activity is associated with the decrease in TERT expression. Using quantitative estimation again we

observed that 2 mM IP6 caused 50% decrease in TERT expression (Fig 7B). Because TERT is generally present in the nucleus, we examined the levels of TERT protein in the nuclear fraction using IP6 treated and untreated TRAMP C2 cells. As we see in Fig. 7C, TERT protein level decreased dramatically in the nuclear fraction. Quantitatively more than 60% of the protein was decreased after IP6 treatment (Fig 7D). We also examined the TERT promoter activity using a 3.3kb TERT promoter-luciferase construct. As we see in Figure 7E, TERT promoter activity was increased approximately 20-fold compared to the basic constructs and IP6 treatment decreased TERT promoter activity in TRAMP C2 cells almost 20-fold. These results again reconfirms our telomerase activity data and reemphasize that IP6 regulates telomerase activity.
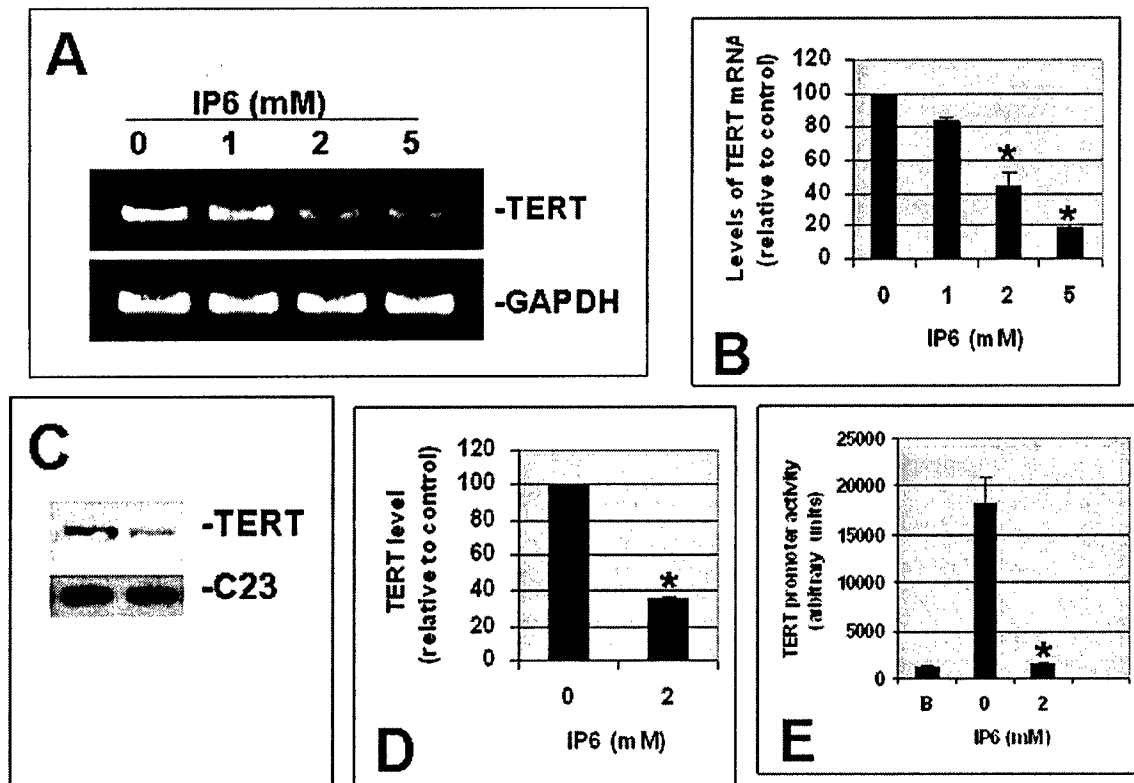

Figure 7. Effects of IP6 on telomerase protein and message in TRAMP prostate cancer cells. A: RT-PCR showing the levels TERT mRNA in IP6 treated and untreated TRAMP C2 cells. B: Quantitative analysis of TERT mRNA in IP6 treated and untreated TRAMP C2 cells. C: Western blot (nuclear extract) showing the levels TERT protein in IP6 treated and untreated TRAMP C2 cells. C-23 (Nucleolin) was used as a loading control. D: Quantitative analysis of TERT protein after normalizing with C-23. E: TERT promoter activity in TRAMP C2 cells with or without IP6 (2mM) for 24h. * indicates significant differences compared to their respective controls.

Since activation of telomerase requires phosphorylation of TERT and Akt is known to phosphorylate TERT, we examined the total and phosphorylated Akt with or without IP6 treatments. As we see in Figure 8, IP6 decreased the phospho-Akt but not the total Akt, suggesting that AKt is deactivated by the IP6. Quantitatively, we also observed that IP6 significantly decreased the activation

of Akt (Fig. 8B). These results suggest that Akt is no longer able to phosphorylate TERT and therefore its translocation to the nucleus.
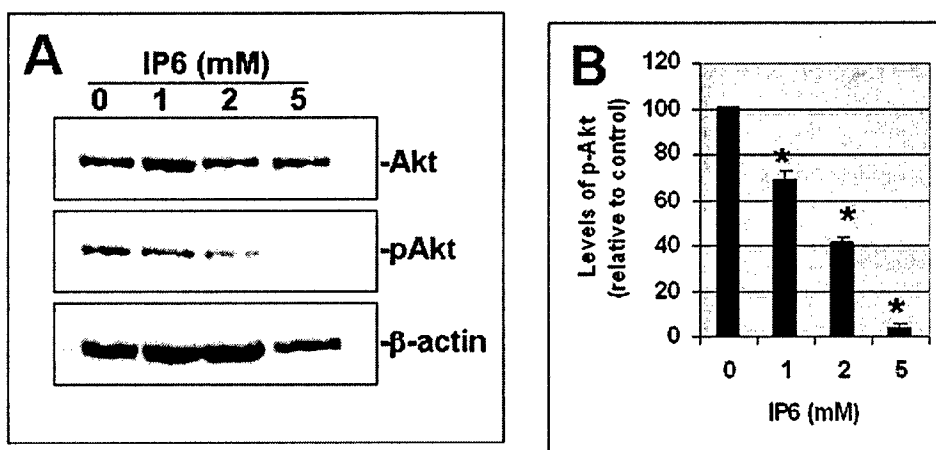

Figure 8. Effects of IP6 on the levels and activation of Akt in TRAMP cells. A: Western blot analyses of total and activated Akt after various doses of IP6 treatments. B: Quantitative analysis of activated Akt with various doses of IP6 in TRAMP C2 cells. * indicates significant differences compared to the respective control.

REPORTABLE OUTCOMES: Two manuscripts 1) Growth inhibition of TRAMP prostate cancer and cancer cells by IP6 is via the down regulation of G0/G1 cell cycle regulators and 2) IP6 inhibits telomerase activity in TRAMP prostate cancer cells are in preparation and will be submitted soon. As soon as we hear the acceptance of these manuscripts, I will send preprints of these manuscripts to US Army Medical Research Material Command. We will also report these findings in upcoming national meetings.

CONCLUSIONS: In my opinion we have progressed quite well and already discovered that IP6 can prevent the development and progression of prostate cancer in TRAMP model. In addition, we also know at least one mechanism by which IP6 inhibits prostate cancer cell growth by inhibiting telomerase activity by deactivating Akt.

REFERENCES: N/A

APPENDICES: none.